THE INFLUENCE OF FOODSTUFFS UPON THE RESPIRATORY METABOLISM AND GROWTH OF HUMAN TUBERCLE BACILLI[1]

R. O. LOEBEL, E. SHORR AND H. B. RICHARDSON

WITH THE TECHNICAL ASSISTANCE OF MURIEL HARRIS

From the Russell Sage Institute of Pathology in Affiliation with the Second (Cornell) Medical Division and the Department of Pathology of Bellevue Hospital and the Department of Medicine, Cornell University Medical College, New York

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INTRODUCTION

The point of view directing these studies can be briefly stated as follows:

In any tuberculous lesion the bacilli must obtain energy for life and growth. The processes involved and their significance for the progress or arrest of a lesion can be investigated by studying, first, the metabolic characteristics of the bacteria, and second, the environment to which they are exposed.

For the sake of concreteness we reproduce figure 1, outlining the physical and chemical features in the environment of the tubercle bacillus. Particular attention may be directed to the blood supply, the monocytes and the caseous material because they may be essential determinants of the quantity of energy available to the bacteria. The detailed experiments and discussion of these factors must appear later in the course of the papers.[2]

[1] This study is part of a group investigation in coöperation with the Research Board of the National Tuberculosis Association. Preliminary reports of this and the succeeding paper have been published in the American Journal of Physiology, 1929, xc, 423, and the Transactions of the National Tuberculosis Association, 1930, xxvi, 196.

[2] For the microphotograph upon which the diagram is based we wish to express our appreciation to Dr. W. Grethman of the Tuberculosis Service of Bellevue Hospital.

METHODS

The organism and its cultivation. The strain of human tubercle bacillus known as H37 was used in these experiments. The cultures were all transplants of the stock material that had been grown from a single organism isolated by Kahn (1929). This has been the parent cell of all the cultures employed by the laboratories associated with the Research Committee of the National Tuberculosis Association. The strain was originally

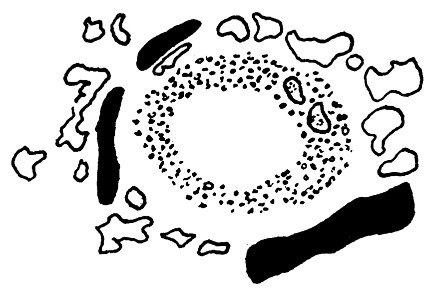

FIG. 1. THE CHEMICAL ENVIRONMENT OF THE TUBERCLE BACILLUS IN THE ANIMAL HOST

Monocytes: Consume oxygen; produce lactic acid; influence pH, undergo caseation.

Caseous material: Coagulated protein; proteose; amino acids; lipoids (20 per cent)—lecithin, cholesterol esters, neutral fat, and calcium soaps; lactic acid; glycogen. Salts: Na, K, Ca, Mg, Fe.

Blood: Glucose, 0.1 per cent; lactic acid, 0.02 per cent; protein, 7.0 per cent; amino acids; lipoids, 0.7 per cent—lecithin, cholesterol, and glycerides of oleic, palmitic, and stearic acids. Salts: Na, K, Ca, Mg, Fe.

cultivated some twenty years ago at the Trudeau laboratory. It is said to be only moderately virulent for the guinea pig. According to Petroff, Branch, and Jennings (1929) this varies with the composition of the medium. Our cultures were maintained in successive transplants on fluid medium of Long (1924) made with the fixed stock reagents controlled by the Research Committee of the National Tuberculosis Association.

This solution has the following composition:

Magnesium sulphate	2.0 grams
Glycerol	100.0 cc.
Asparagin	10.0 grams
Ammonium citrate	10.0 grams

Potassium acid phosphate	6.0 grams
Ferric ammonium citrate	0.1 gram
Sodium carbonate	4.2 grams
Sodium chloride	4.0 grams
Distilled water to	2000.0 cc.

The pH was adjusted to 7.2 with the help of sodium carbonate.

Value of starvation. Early in these studies it was found that removal of tubercle bacilli from Long's to a non-nutrient solution did not result in a prompt depression of the respiration. Deprival of foodstuff for several days was necessary.

Non-nutrient solutions employed. For this purpose the surface growth of bacilli was floated upon sterile solutions in Erlenmeyer flasks. We employed 50 cc. of a solution containing 0.9 per cent sodium chloride and M/150 sodium phosphate buffers at a pH of 7.4. After several days on this fluid the bacteria were transferred to the respiratory vessels for metabolic study.

Depending upon the purpose of the experiment these contained one of the following solutions:

1. Long's medium, unchanged or with the omission of one or more ingredients.
2. A 0.9 per cent solution of sodium chloride buffered with sodium phosphate as just described.
3. Distilled water similarly buffered.

The last two were employed with or without the addition of the foodstuff to be tested.

Apparatus for measuring oxygen consumption. We used Barcroft-Warburg manometers, of which a full description will be found elsewhere (Richardson, 1929). Figure 2 is a diagram of the manometer as modified for these experiments. The left limb of the manometer being in the same plane as the right, does not appear in the diagram. At the U-shaped bend both limbs communicate with each other and also with a rubber reservoir containing Brodie's manometer fluid. In operation the vessel was submerged in a water bath at 37.5°C. while the manometer was on the outside of the thermostat except where it was attached to the vessel by means of a ground glass connection.

Inasmuch as contact of the organisms with the stem of the manometer would increase the hazard of dissemination when the two parts were detached from each other, the stem of the manometer was constructed to fit on the outside of the vessel. The whole system mounted on a board was supported on a shaker which accommodated seven such units for the purpose of producing a to-and-fro motion. The shaking brought about prompt diffusion and equilibrium between the gases of the fluid in the vessel and the space above it. The vessels were of about 20 cc. capacity. They usually contained 2.2 cc. of fluid. Of this,

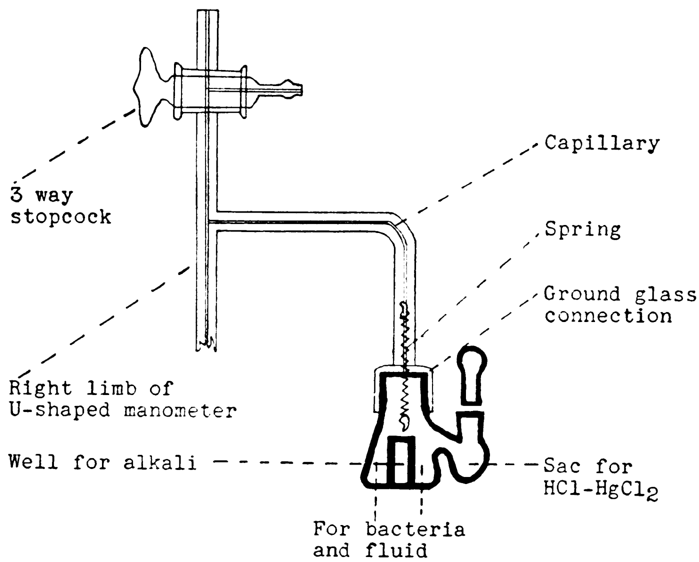

FIG. 2. MODIFIED BARCROFT-WARBURG MANOMETER

Scale: |——| = 2.5 cm.

0.2 cc. was N KOH in the central portion which served to absorb carbon dioxide produced as the result of respiration. When the bacteria in the 2 cc. of fluid consumed oxygen there was a fall in pressure as recorded by the manometer. By multiplication with an easily derived factor this was converted to cubic millimeters of oxygen consumed. Results were expressed as cubic millimeters per moist milligram of bacteria per hour.

Before transfer to the vessels the bacteria were placed for a few minutes on filter paper until the adherent moisture was absorbed. Then they were transferred to small crescentic pieces of thin watch glass which were introduced into stoppered tares and weighed. An amount of bacteria sufficient to cause a change in pressure of 25 to 100 mm. per hour was chosen. The slip of glass containing this quantity was placed at the bottom of the Warburg vessels. Here the clumps were disintegrated with the aid of a wire because it was found that such comminution led to greater uniformity in respiration. After this, the vessels were attached to the manometers, suspended in the water bath, shut off from the outside air by turning the stop-cock of the manometer, and then shaken for about twenty minutes in order to establish equilibrium. The first two readings occurred at fifteen-minute intervals. Later ones were taken every half hour. In every experiment the oxygen consumption was determined in duplicate or triplicate.

Method of measuring the influence of foodstuff upon the starved bacteria. In experiments with the various foodstuffs there was included a determination of the oxygen consumption of the starved bacteria in a saline phosphate solution. With these figures one could easily calculate how much the foodstuff had increased the respiration above the "starvation" level. With sodium lactate the respiratory studies were supplemented by the chemical determination of the lactate disappearing from the solution.

SOURCE AND PREPARATION OF FOODSTUFFS

Carbohydrates and allied compounds. Of the foodstuffs employed in these experiments, glucose, racemic sodium lactate, mannose and levulose were obtained from Kahlbaum. Since the sodium lactate varied in concentration and was generally somewhat acid it was necessary to determine the concentration by chemical analysis and to neutralize with N/10 NaOH before using. The glycogen, inositol, and arabinose were Eimer and Amend's C.P. Glycerol C.P. was obtained from Mulford.

Fatty acids. Oleic, stearic and palmitic acids were C.P., Eimer and Amend. They were saponified with sodium hydroxide and then salted out with sodium chloride. The oleate went readily into solution, while the other two formed a suspension. After warming and shaking they were all diluted 1:10 with phosphate at pH 7.4. The molarity of the phosphate was higher than usual, i.e., M/75, in order to keep down the pH of the solutions. Tested at room temperature this was between 7.4 and 8.0 for the various soaps.

Fats. In order to test the influence of an animal fat we employed cream of 40 per cent fat content. This was washed repeatedly with distilled water, centrifuged, and made up to a 1 per cent suspension. Finally, it was diluted with 9 times its volume of M/75 phosphate solution in water or in physiological saline. Owing to irregularities in suspension the material probably had less than the indicated amounts of fat. In addition, it contained at the time of one experiment 0.0005 per cent of lactic acid, no reducing sugar, and 0.005 per cent of nitrogen.

Serum and serum fat. The serum used for these experiments was obtained from normal or arthritic patients by venepuncture and centrifugation under sterile precautions. Several specimens were then pooled. By means of the addition of 2 or 3 drops of N/10 HCl per cubic centimeter of serum the pH was brought to 7.4 before use in the Warburg vessels. In order to extract the fat, 15 cc. were precipitated with 250 cc. of alcohol-ether in a mixture of 3:1. After filtration the filtrate was evaporated down to 3 or 4 cc., at which time no odor of alcohol or ether was perceptible. The residue was taken up by the usual solution of phosphate and saline at pH 7.4 to a final volume of 15 cc., i.e., the original quantity of serum.

Control of glucose and lactate in serum. In two experiments the lactic acid and glucose content of the extract were determined. Then control experiments, where these quantities were added to a solution of saline phosphate, were included in the experiments on serum fat. In two others, those interfering substances were removed by dialysis for three days during which the washings were changed twice. It was further necessary to concentrate the final solution about 40 per cent by evaporation in order to restore it to the original volume.

Lecithin. Lecithin from eggs (Eimer and Amend) was made into a 0.5 per cent suspension in a solution of saline and phosphate. Except in the cases where the organisms were suspended in a thin film of solution in 50-cc. Erlenmeyer flasks for incubation over night neither the serum fat nor the lecithin was autoclaved.

Caseous material. This was obtained from a tuberculous kidney at operation. The lesions did not communicate with the ureters so that the chances of secondary infection were greatly diminished and, in fact, cultures on ordinary media were sterile. In one set of chemical determinations we obtained the following figures: Lactic acid, 0.0006 per cent; glucose, none detected; and nitrogen, 0.14 per cent (expressed as protein).

The material was kept on ice until ready for use when it was diluted with 9 times its volume of buffered saline solution.

DISCUSSION OF METHODS

Respiratory experiments contrasted with cultivation or inoculation experiments. It is to be noted that the method which we have employed gives the average respiration per milligram of the whole mass of bacteria under investigation.

This contrasts with the methods which have generally been used for studying the metabolism of the organism. These depend upon the growth of the organism during the experimental period or upon the death of an animal by inoculation. With both of these procedures the survival of a few bacteria may be sufficient to give positive results.

Other types of measurement of the gaseous metabolism. There have been few studies of the respiratory metabolism of the tubercle bacillus. Novy and Soule (Novy, Roehm and Soule, 1925; Novy and Soule, 1925 and 1927) measured the consumption of oxygen and the production of carbon dioxide by a culture of tubercle bacilli over a period of weeks, during which time active growth and in consequence, considerable alteration in bacterial mass occurred. Merrill (1930, 1931a and b) has obtained figures for the total carbon dioxide produced during growth of cultures of acid-fast bacteria.

Although such experiments are of great value they are subject to several limitations. The metabolic transformations cannot be expressed per unit of organism. Nor can those changes be studied which may occur in the respiratory metabolism or in the number of viable organisms during the several weeks which comprise the period under observation.

Finally the heaping up of bacteria during growth adds a complicating factor due to the distance of the organisms from the foodstuff.

Control of contamination during respiratory measurements. In the experiments reported in this paper, the period during which respiration was measured was only a few hours. This made possible a great gain in simplicity of manipulations, for it became unnecessary to maintain strictly aseptic conditions in the Warburg vessels. In 13 experiments to ascertain whether errors arise due to contamination, two control vessels were set up, containing Long's solution but no bacteria. One vessel had KOH in the well, while the other had none. The excellent agreement between the two indicated that no carbon dioxide was being produced by respiration. For, if it had been, a negative pressure would have resulted when the carbon dioxide was absorbed by the alkali. Among the many internal evidences that no further precautions were necessary may be mentioned the fact that tubercle bacilli after several months of culture showed no respiration although the manipulations and chances for contamination were exactly the same.

Control of oxidation of foodstuffs—either spontaneous or by dead tubercle bacilli. Another possible source of error was the autoxidation of foodstuffs. The various soaps, serum fat, and lecithin were investigated and found to have no oxygen uptake. In the case of the soaps these control studies were also conducted in the presence of tubercle bacilli killed by autoclaving. After such treatment the organisms did not respire.

Does shaking influence respiration? In attempting to apply data obtained by the Warburg technique to conditions in culture and in the body one other question immediately presented itself. Was the respiration of the bacteria influenced by the shaking of the Warburg vessel? It was found that absence of shaking was associated with a diminution of oxygen uptake which varied from 25 to 55 per cent. For two reasons this difference might have been more apparent than real. It might have been due to a failure of gaseous equilibrium in the unshaken vessels, or to the tendency of the unshaken bacteria to form large macroscopic clumps. Inasmuch as the organisms in the body do not generally form such aggregates it may well be that the respiration measured by our routine procedure is closer to the value obtaining in the body. However it would be unprofitable to pursue this discussion since the environment of the tubercle bacilli in the body may easily cause much wider differences in respiration than those which have been considered here.

RESULTS

Influence of age of culture and of alterations in culture medium upon respiration. In order to study the fluctuations of oxygen consumption with age a single culture was used to seed a large number of Erlenmeyer flasks of 200 cc. capacity, each containing 50 cc. of Long's medium. Every week, three flasks were removed from the incubator, an adequate amount of the floating film of microörganisms was lifted out with a very large wire grid, and the clear underlying culture medium was aspirated. The bacteria were weighed and then re-united with 3 cc. of the aspirated solution in a Warburg vessel, where the respiration was studied. The figures are given in curve I of figure 3.

Although it was on the eighth day that the highest figure was obtained, 2.7 cu. mm., this was almost equalled on the fifteenth day, after which there was a rapid falling off. It may be mentioned that on the fifteenth and twenty-second day the organisms consumed the same amount of oxygen on fresh Long's as upon their old Long's medium.

The results were much the same, except for a longer persistence of respiration, when the experimental procedure was altered by removing

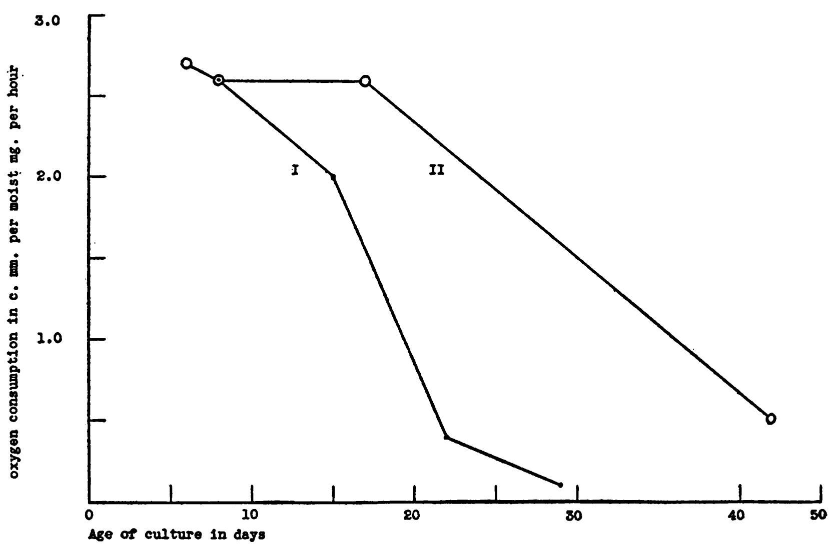

FIG. 3. THE EFFECT OF AGE UPON THE RESPIRATION OF H37 IN LONG'S MEDIUM

areas of growth from the same flask on successive occasions (see curve II of figure 3). The uniformity of the respiration in different cultures of the same age and generation was striking, the deviation from the average being less than 0.1 cu. mm., except in one instance when it was 0.3 cu. mm., from an average of 2.6 cu. mm. The level in the first two weeks of growth in the large group of subsequent experiments varied between 2.7 and 1.0 cu. mm. usually being nearer the lower figure. The latter represents a considerable utilization of oxygen, twice as much, for instance, as that recorded for the resting muscle of the dog, or one-third that of the dog's kidney (Richardson, Shorr and Loebel, 1930) which has a high rate. However, it is only one-twentieth to one-fiftieth as high as the respiration of pneumococci (Finkle, 1931) that have grown on glucose broth and have been suspended in a buffered solution of 0.5 per cent glucose.

The effect of the various constituents of Long's solution upon respiration. Starting with the usual Long's solution, one or more ingredients were omitted; or else starting with a buffered solution of saline-phosphate, glycerol was added. On these solutions of varying composition, the

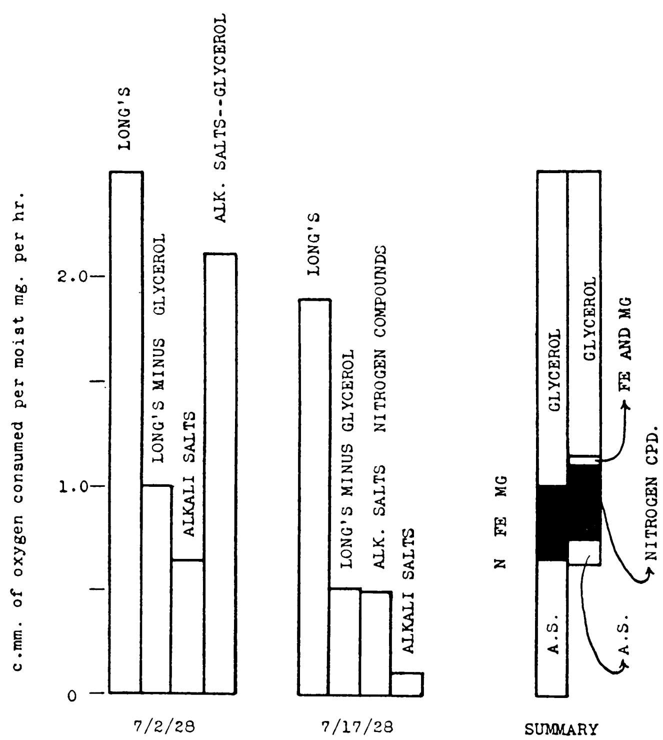

FIG. 4. THE EFFECT OF VARIOUS CONSTITUENTS OF LONG'S SOLUTION UPON THE RESPIRATION OF H37

respiration of organisms from the same culture was studied after a few days starvation. The results are shown graphically in figure 4. In the upper graph is shown successively the effect of the absence of glycerol, of the absence of nitrogenous constituents, and of the addition of glycerol. The summary reveals that glycerol was responsible for about four-fifths of the increase in respiration above the fasting level, and the nitrogenous constituents for one-fifth. Incidentally it may be remarked that the increase caused by the latter is the usual share of the total contributed by protein in the ordinary diet of man. The iron and magnesium together had a negligible effect in short experiments.

The low values in the salt solutions will be considered in greater detail in a following paper. At this point we should like, merely, to emphasize the extreme usefulness of the period of "starvation." It depressed the respiration so that the effect of added foodstuff was not masked by reserves which the organism had itself stored.

Effect of varying concentrations of glycerol upon respiration. Glycerol in a concentration of 5 per cent maintained respiration for four or five hours, both as an ingredient of Long's solution and of buffered saline solution; in fact, in the majority of instances the later hours showed an increase over the first hour. This contrasted with the oxygen consumption without food which diminished sharply at a rate averaging 16 per cent per hour for the whole group of experiments.

It is interesting to inquire whether glycerol is effective at concentrations lower than 5 per cent which is employed to obtain abundant growth. Table 2 shows that in solutions of 0.05 and 0.02 per cent the respiration was still considerable as compared to that in 5 per cent glycerol. Other experiments showed that even at 0.005 per cent a distinct action was apparent. Energy was available far below the concentrations necessary for maximal or even minimal growth.

Effect of varying concentration of glucose upon respiration. Glucose (see tables 1 and 2) is known to be a partial substitute for glycerol in the cultivation of the tubercle bacillus. Table 1 shows the effect of this and other saccharides as well as sodium lactate and glycerol upon respiration. Glucose caused a definite increase, and also permitted a certain amount of growth when substituted for the glycerol of Long's solution. It is worthy of note that the influence of glucose seemed to be detectable down to 0.1 per cent but not at lesser concentrations.

Effect of levulose and other saccharides. (See table 1.) Levulose had no evident effect upon the respiration and permitted no growth. In two experiments glycogen supported a respiration 3 times as high as the fasting level. However, the absolute value was very low. Mannose was without effect. Inositol and arabinose, which Roberts and Anderson (1931) have found in the water soluble fraction of the purified wax of H37 gave no significant increase.

Effect of varying concentration of sodium lactate. (See tables 1 and 2.) Sodium lactate, at a concentration of 5 per cent, caused an increase in the respiration of even greater magnitude than glycerol but did not

TABLE 1

The effect of various foodstuffs upon the respiration of H37

DATE	FOOD	CONCENTRATION	PREVIOUS MILIEU: Time in Long's solution	PREVIOUS MILIEU: Time of starvation	DURATION OF OBSERVATION	OXYGEN CONSUMPTION PER MOIST MILLIGRAM IN FIRST HOUR: In non-nutrient solution*	In food of column 2	In 5 per cent glycerol	In Long's solution	Comparison of food with† 5 per cent glycerol	Comparison of food with† Long's solution	AVERAGE CHANGE IN RESPIRATION IN FOOD OF COLUMN 2
		per cent	*days*	*days*	*hours*	*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*	*per cent*	*per cent*	*per cent per hour*
1/11/29	Glucose	5	16	2	1½	1.08	1.34	1.83		35		
1/15/29	Glucose	5	16	6	1½	0.61	0.88	1.5		29		
3/19/29	Glucose	5	14	10	2½	0.74	0.74	1.35		0		+33
4/15/29	Glucose	5	15	2	2	0.94	0.90	1.2		0		0
4/14/30	Glucose	5	14	10	2	0.24	0.56	1.2		33		+32
1/11/29	Levulose	5	16	2	1½	1.08	1.15	1.83		9		
1/15/29	Levulose	5	16	6	1½	0.61	0.49	1.5		0		
4/15/29	Levulose	5	15	2	2	0.94	0.73	1.2		0		−1
5/16/30	Mannose	5	14	13	2	0.10	0.10	1.50		0		−27
5/22/30	Mannose	5	14	19	3	0.07	0.13	0.73		9		−38
5/16/30	Inositol	5	14	13	2	0.10	0.09	1.50		0		+67
5/22/30	Inositol	5	14	19	3	0.07	0.11, 0.42	0.73				
7/14/30	Inositol	5	16	11	2	0.20	0.20	0.93		0		+50

5/16/30	Arabinose	5	14	13	2	0.10	0.16	1.50		4		+70
5/22/30	Arabinose	5	14	19	3	0.07	0.32, 0.10	0.73		—		
7/14/30	Arabinose	5	16	11	2	0.20	0.25	0.93		7		0
5/16/30	Glycogen	5	14	13	2	0.10	0.33	1.50		16		−15
5/22/30	Glycogen	5	14	19	3	0.07	0.26	0.73		29		−23
1/11/29	Sodium lactate	5	16	2	1½	1.1	2.1	1.8				
1/15/29	Sodium lactate	5	16	6	2½	0.6	2.4	1.5				−6
3/19/29	Sodium lactate	5	14	10	1½	0.96	1.1	1.35				+7
4/15/29	Sodium lactate	5	15	2	2	0.74	1.6	1.2				+13
7/17/28	Asparagin and NH_4 citrate	0.5 0.5	16	10	1	0.12	0.47		1.88		20	
2/27/30	Sodium oleate	0.1	20	2	3⅔	0.98	1.11		1.4		31	−40
3/ 6/30	Sodium oleate	0.1	13	3	2	0.72	1.96		1.82		111	−6
3/11/30	Sodium oleate	0.1	18	3	5⅓	0.71	1.75	1.29		179		−12
6/20/30	Sodium oleate	0.1	14	8	2¼	0.53	0.30	1.28		0		−73
2/27/30	Sodium palmitate	0.1	20	2	3⅔	0.98	0.99		1.4		0	+17
3/ 6/30	Sodium palmitate	0.1	13	3	2	0.72	1.75		1.82		94	−2
3/11/30	Sodium palmitate	0.1	18	3	5⅓	0.71	1.62	1.29		157		0
6/20/30	Sodium palmitate	0.1	14	8	2¼	0.53	2.00	1.28		197		0

* The non-nutrient solution was 0.9 per cent saline brought to pH 7.4 by means of M/150 phosphate buffers.

† This is the ratio calculated by using the following equation:

$$\frac{\text{Respiration in food of col. 2} - \text{Respiration in non-nutrient solution}}{\text{Respiration in glycerol 5 per cent (or Long's solution)} - \text{Respiration in non-nutrient solution}}$$

e.g., for the glucose experiment of 1/11/29, $\frac{1.34 - 1.08}{1.83 - 1.08} = 35$ per cent

TABLE 1—*Concluded*

DATE	FOOD	CONCENTRATION	PREVIOUS MILIEU: Time in Long's solution	PREVIOUS MILIEU: Time of starvation	DURATION OF OBSERVATION	OXYGEN CONSUMPTION PER MOIST MILLIGRAM IN FIRST HOUR: In non-nutrient solution*	In food of column 2	In 5 per cent glycerol	In Long's solution	Comparison of food with†: 5 per cent glycerol	Comparison of food with†: Long's solution	AVERAGE CHANGE IN RESPIRATION IN FOOD OF COLUMN 2
		per cent	*days*	*days*	*hours*	*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*	*per cent*	*per cent*	*per cent per hour*
2/27/30	Sodium stearate	0.1	20	2	$3\frac{2}{3}$	0.98	1.60		1.4		148	+21
3/ 6/30	Sodium stearate	0.1	13	3	2	0.72	2.18		1.82		133	−4
3/11/30	Sodium stearate	0.1	18	3	$5\frac{1}{3}$	0.71	1.61	1.29		155		−12
3/31/30	Serum	Whole	14	10	1	0.15	1.7		1.25		141	
4/18/30	Serum	Whole	14	14	5	0.16	1.69		1.32		132	−8
5/30/30	Serum	Whole	23	11	3	0.36	1.5		0.78		272	−10
10/31/30	Serum	Whole	15	9	2	0.28	1.97	1.23		178		0
11/17/30	Serum	Whole	14	12	$2\frac{1}{2}$	0.05	2.02	0.86		243		+2
11/18/30	Serum	Whole	14	13	3	0.09	1.64	0.82		212		−4
3/31/30	Serum	10	14	10	1	0.15	0.56		1.25	37		
5/30/30	Serum fat	As in serum	23	11	3	0.36	0.06	1.23	0.78		0	+42
10/ 8/30	Serum fat	As in serum	15	11	$4\frac{1}{2}$	0.14	1.38	0.94		155		−12
10/31/30	Serum fat	As in serum	15	9	2	0.28	1.93			178		0
11/17/30	Dialyzed serum fat	As in serum	14	12	$2\frac{1}{2}$	0.05	0.65	0.86		74		
11/18/30	Dialyzed serum fat	As in serum	14	13	3	0.09	0.79	0.82		96		+27

10/ 8/30	Lecithin	0.5	15	11	$4\frac{1}{4}$	0.14	0.75	0.94		76		−13
10/11/30	Lecithin	0.5	15	14	3	0.06	0.56	0.87		62		−21
10/31/30	Lecithin	0.5	15	9	2	0.28	1.18	1.23		95		−3
11/ 1/30	Lecithin	0.5	15	9	2	0.31	1.05					+10
4/24/30	Milk fat	0.1	14	12	1		1.03					
4/25/30	Milk fat	0.1	14	13	4	0.15	0.71	1.15		56		−24
5/ 1/30	Milk fat	1.0	15	12	3	0.26	0.61	1.25		35		−16
5/ 1/30	Milk fat	0.1	15	12	3	0.26	0.29	1.25		3		−12
10/ 8/30	Milk fat	1.0	15	11	$4\frac{1}{4}$	0.14	0.24	0.94		13		+55
3/18/30	Caseous material	10	15	4	$3\frac{1}{4}$	0.86	1.56					−23
3/20/30	Caseous material	10	15	6	4	0.48	1.81		1.45		137	−2
4/18/30	Caseous material	10	14	14	5	0.16	1.72		1.32		134	−30
7/28/30	Caseous material	10	13	5	$3\frac{3}{4}$	0.30	1.0		1.68		51	−16

TABLE 2

Effect of diminishing concentration of foodstuff upon the respiration of H37

DATE	DURATION OF EXPERIMENT	PREVIOUS MILIEU: Time in Long's solution	PREVIOUS MILIEU: Time of "starvation"	OXYGEN CONSUMPTION PER MOIST MILLIGRAM PER FIRST HOUR: In 0 per cent	In 5.0 per cent	In 1.0 per cent	In 0.1 per cent	In 0.08 per cent	In 0.05 per cent	In 0.03 per cent	In 0.02 per cent	In 0.01 per cent	In 0.005 per cent
A. In glycerol* of varying percentages													
	hours	*days*	*days*	*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*
3/ 8/30	2	22	1	1.19	1.69	1.56	1.40						
3/13/30	5	18	5	0.20	0.88	0.71	0.73						
4/14/30	2	14	10						0.94		0.76		
4/23/30	4	14	11	0.25	1.12		1.17						
4/25/30	4	14	13	0.15	1.15							0.38	0.27
7/15/31	1	25	6	0.25					0.67		0.51	0.38	0.35
B. In glucose* of varying percentages												Control in: 5 per cent glycerol	Control in: Long's solution
4/14/30	2	14	10		0.56	0.58	0.16					1.20	
4/18/30	$5\frac{1}{4}$	14	14	0.16					0.29				1.32
4/23/30	4	14	11	0.25			0.45					1.12	
5/30/30	3	23	11	0.36						0.17			0.78
7/18/31	$1\frac{1}{2}$	21	4	0.37			0.49		0.39				1.48
6/ 7/32	2	20	12	0.09	0.30	0.29	0.07					0.80	
6/14/32	3	18	4	0.20	0.56	0.45	0.28					0.95	
C. In sodium lactate* of varying percentages													
4/ 5/30	1	15	7	0.19	2.76	2.67	0.96		0.68		0.44		
4/18/30	$5\frac{1}{4}$	14	14	0.16				0.43					1.32
4/23/30	4	14	11	0.25			0.83					1.12	
5/30/30	3	23	11	0.36				0.40					0.78
6/ 5/30	2	23, 16†	17	0.10		0.74							
7/ 8/30	3	17	19	0.16			0.41						
7/18/31	$1\frac{1}{2}$	21	4	0.37					0.64		0.49		1.48

* These foodstuffs were added to the non-nutrient solution consisting of the usual saline and phosphate.

† Two cultures were lumped.

promote growth. It must be added, however, that with a strength of 1 per cent there was a consistent, if slight, increase in the area of the transplant. This did not persist after transfer to a fresh solution of lactate. 1 per cent sodium lactate was nearly as effective in supporting respiration as higher concentrations. Even 0.02 per cent exerted a definite stimulation.

Comparison of chemical and gasometric estimation of the utilization of lactic acid. A comparison of the oxygen uptake and the simultaneous disappearance of lactic acid is contained in the following protocols.

July 8, 1930

1. Lactic acid in 4 cc. of solution = 4.02 mgm.
2. Lactic acid per milligram of H37 = 0.0015 mgm.
3. Lactic acid in 4 cc. of solution + 339 mgm. of H37:
   a. At end of equilibration, i.e., beginning of experiment = 4.53 mgm. [calculated from (1) and (2)].
   b. At end of $4\frac{1}{2}$ hours of respiration = 3.32 mgm.
   Difference = lactic acid disappearing in $4\frac{1}{2}$ hours = 1.21 mgm.

The total oxygen consumption in $4\frac{1}{2}$ hours was equivalent to 1.03 mgm.

The oxygen consumption in lactate minus that in a non-nutrient solution was equivalent to 0.67 mgm.

July 21, 1930

1. Lactic acid in 4 cc. of solution = 3.78 mgm.
2. Lactic acid per milligram of H37 = 0.00097 mgm.
3. Lactic acid in 4 cc. of solution + 303 mgm. of H37:
   a. At end of equilibration, i.e., beginning of experiment = 4.11 mgm. [calculated from (1) and (2)].
   b. At end of 5 hours of respiration = 3.08 mgm.
   Difference = lactic acid disappearing in 5 hours = 1.03 mgm.

The total oxygen consumption was equivalent to 1.08 mgm.

The respiration in lactate minus that in a non-nutrient solution was equivalent to 0.60 mgm.

On the basis of the total oxygen consumption all of the lactic acid disappearing could be explained by its complete oxidation or, conversely, all of the respiration would be due to the lactic acid. However, there is no certainty that the material which was oxidized in the non-nutrient solutions was entirely displaced by the lactate. If the results are computed on the assumption that no such displacement occurred, then only two thirds of the lactic acid which disappeared was accounted for by oxidation and the rest might have disappeared by being synthesized to some other compound or by incomplete combustion.

The effect of fatty acid upon respiration. Is glycerol the only fraction of the fat molecule which can supply energy, or can the fatty acid portion of the molecule also serve the same function? As evidenced by table 1 the oxygen consumption even with a concentration of 0.1 per cent of a soap of the higher fatty acids, was considerably higher than with Long's solution or with glycerol in a concentration of 5 per cent. With oleate there was a falling off in respiration of 30 per cent per hour. With the other two there was little or no change (see fig. 5). This distinction was borne out by exposing the bacilli to soaps in the same strength for 18 hours in dyer's beakers of 600 cc. capacity. To prevent the prompt sinking which otherwise took place they were floated on cork discs wrapped with filter paper to keep the bacilli wet. As had

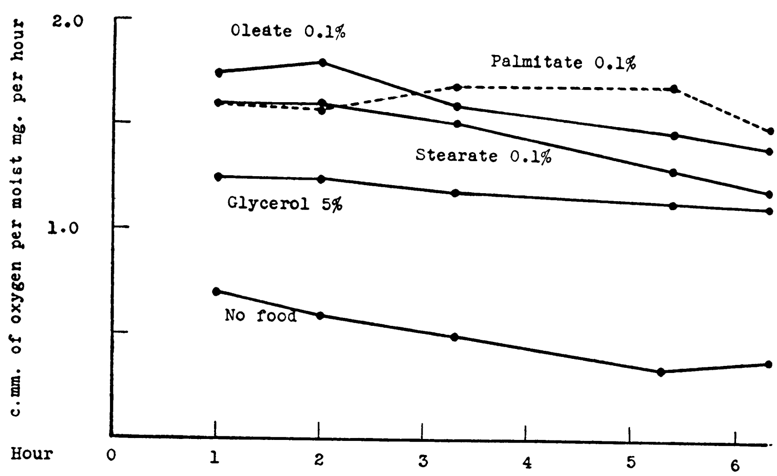

FIG. 5. THE EFFECT OF SOAPS OF THE HIGHER FATTY ACIDS UPON THE RESPIRATION OF H37

been previously shown by others, the presence of cork and filter paper was not injurious in control experiments on Long's solution, where the customary profuse growth took place. When a solution of oleate was used to suspend the organisms for 18 hours and then to test the respiration, it was found after 18 hours that the power to consume oxygen had been lost, whereas a similar procedure with palmitate showed that the respiration was as high after 18 hours as after 1 hour.

Effect of fatty acids upon growth. To test the inhibition of growth, organisms were floated upon suspensions of stearate in a concentration of 1:10,000. After 4 hours the bacteria were transferred to the surface of fluid Long's medium. In 14 days the transfers from stearate multiplied to form a thin sheet covering one-third of the surface.

Effect of fat. Inasmuch as both glycerol and fatty acid were available as sources of energy the question arose whether fat itself would stimulate respiration. Table 1 shows a very definite stimulation of respiration by cream fat. The oxygen consumption was fairly well maintained, the change being minus 6 to 8 per cent per hour as compared to the first hour. On Long's medium in which milk fat was substituted for glycerol to make a concentration of 0.1 per cent there was no growth.

Effect of serum. (See table 1 and fig. 6.) Here the respiration was higher than in 5 per cent glycerol or in Long's solution. There was no tendency to diminish from hour to hour. Even when the serum was diluted with 9 times its volume by means of saline-phosphate it caused

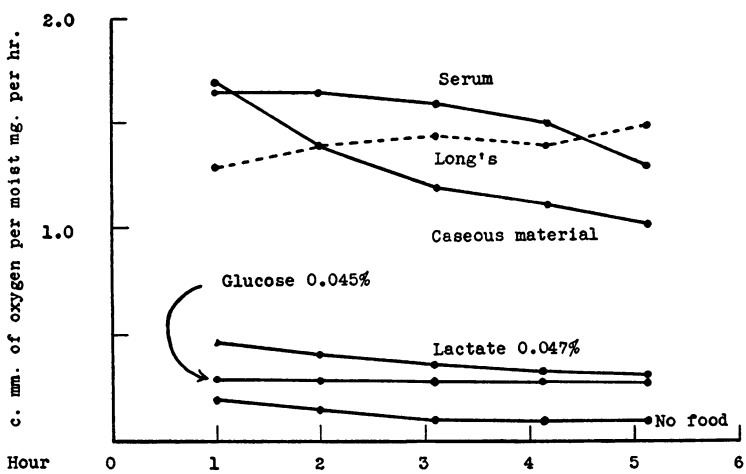

FIG. 6. THE EFFECT OF SERUM AND CASEOUS MATERIAL UPON THE RESPIRATION OF H37

a rise to 3 times the fasting level. Very little of this was due to the presence of glucose or lactate in the serum, as was shown by controls in which the same amounts of glucose or of lactate were added to saline-phosphate solution.

Importance of serum fat. (See table 1.) Could it have been due to serum fat? In 4 out of 5 experiments with that material the respiration was stimulated to 6 or 10 times the fasting value. This compared favorably with the stimulation by glycerol, but lagged behind whole serum. In one experiment the increased respiration brought about by serum fat was maintained for a long time. This material was made free of glucose and lactate by dialysis. Then it was used, first, to suspend tubercle bacilli in an Erlenmeyer flask over night in the incubator and on the following day to suspend the same organisms in Warburg vessels where their respiration was measured. It was found that even after this prolonged contact (19 hours) the serum fat stimulated as much as usual.

Effect of lecithin. (See table 1.) If one next examines the three experiments with lecithin it will be seen that the latter caused a five-fold increase in the respiration. It was as effective as glycerol. Even after tubercle bacilli had been in contact with lecithin for 19 hours in an Erlenmeyer flask they showed this heightened respiration when studied in Warburg vessels containing lecithin.

Effect of caseous material. (See table 1 and fig. 6.) In all 4 of the experiments with caseous material the respiration was stimulated considerably above the "fasting" level and in two out of the three experiments where the comparison was made, the stimulation even exceeded that by Long's solution.

DISCUSSION

Increased respiration as a criterion of food utilization. From the data it is obvious that a number of foodstuffs caused an increase in oxygen consumption. Was this entirely due to the oxidation of the food or did the latter merely act as a general stimulus under whose influence the bacteria oxidised food they had carried over from Long's culture medium? In many experiments the bacteria were starved on saline phosphate for so long and the fasting level of the respiration was so low that it is extremely improbable that a considerable amount of food could have been transferred with them into the respiratory vessels. Nor is non-specific stimulation a likely explanation of the numerous instances where the presence of food was associated with an oxygen consumption that was much higher than the fasting level from the beginning and was maintained at a high figure from hour to hour. As can be seen from table 1 the respiration with food was nearly always at 2 or 3 times the fasting level, and occasionally, 10 times as great. On the whole, these levels were well maintained. In Long's medium, in glycerol and in sodium lactate there was even a tendency to increase from hour to hour. The soaps varied in this respect. Oleate caused a decline of 30 per cent per hour whereas palmitate and stearate exhibited no change. The last one mentioned, in fact, supported respiration after 18 hours at the same level as in the first hour. The decline with milk fat averaged 1 per cent, with serum 2 per cent, and with caseous material 8 per cent. The average decline in oxygen consumption without food was 16 per cent.

Besides the maintenance of respiration one can obtain evidence that a foodstuff is a source of energy by observing whether it supports growth. With many of the substances such experiments were performed in addition to the respiratory determinations. They will be discussed in the text below.

The most conclusive evidence can be furnished by comparing the increase in oxygen consumption with the amount of the food which disappears. The latter can be determined chemically. When this experiment was performed with sodium lactate an exact correspondence was found. In view of the facts, it is likely that in all cases the increases of respiration represented the utilization of the food which had been added.

The rôle of glycerol. For a variety of reasons, glycerol is perhaps the most important material which was studied.

In the early days of cultivation of the tubercle bacillus glycerol was added to culture media as a hydrophilic agent (Nocard and Roux, 1887). The fact that it was essential for the abundant growth of many strains indicated that it was much more than this. Even with the unsatisfactory methods available for the determination of glycerol C. Siebert (1909) was able to demonstrate that large amounts disappeared during the growth of a culture. Novy and Soule (1925) measured the total oxygen consumption and carbon dioxide production of culture tubes of tubercle bacilli from the time of inoculation to a month or more of age. The respiration was about 4 times as great with agar broth containing glycerol as with a similar medium containing glucose or with rabbit's serum.

Inasmuch as the growth was also more abundant in the first instance it is impossible to state whether the respiration per unit of weight was different. The fluctuations which may have occurred at the various age periods were not measured. Utilization of the foods in question was indicated by respiratory quotients which approximated the theoretical values for glucose and glycerol. With serum, the quotient was 0.90. There is however some difficulty in accepting this simple explanation. In order to obtain a medium on which the bacteria grew it was necessary to use broth in addition to glycerol. The oxidation of nutrient material in the former might easily cause alterations in the respiratory quotient. The occurrence of metabolic transformations other than complete oxidation to carbon dioxide and water must also be considered. These complicating factors may explain why in later work with the bovine bacillus (Novy and Soule, 1927) obtained respiratory quotients with glycerol which were somewhat higher than the theoretic. In spite of this criticism it is very hard not to be impressed by the almost theoretic quotients for glycerol and glucose which they obtained. There is great likelihood that with the human and bovine tubercle bacillus in glycerol broth, the oxidation of glycerol is complete and altogether overshadows, in amount, the oxidation of other foodstuffs.

Regardless of whether glycerol is oxidised completely or not, the experiments which we have performed in the Warburg vessels demonstrate that glycerol supplies a very considerable amount of energy. The application of this fact to the growth of the tubercle bacillus in man is not entirely obvious.

Long (1930) pointed out in a recent Harvey lecture the discrepancy between the high concentration of glycerol necessary for growth and the low concentration in the circulating blood. Whereas 5 per cent is necessary for abundant growth of H37 and 0.5 per cent for the minimum, the concentration in human blood is probably very small. In pig's blood Schmitz (1912) found 0.004 per cent. His figure for ox blood was 0.002 per cent which compares with 0.007 per cent obtained by Tangl and Weiser (1906).

It will be recalled that in our experiments distinct increases of respiration were obtained with 0.005 per cent. Moreover, reference to table 2 makes it apparent that respiration was at the same level whether the concentration was 5 or 0.1 per cent, so that a low concentration, supplied continuously might furnish as much energy as higher concentrations. For that reason the failure of H37 to grow in a culture flask containing less than 0.5 per cent glycerol does not rule out the possibility of growth at low concentrations of glycerol in a circulating blood stream. The cultivation experiments have even less weight against the respiratory experiments in answering the question whether very low concentrations of glycerol may not furnish energy for survival if not for growth. Before leaving the subject it may be mentioned that it is by no means clear that in the body the organisms depend upon the blood stream directly for glucose or any other material which they may utilize. It is well known that tubercle bacilli are generally found in cells, usually of the monocytic series. While here, the concentration of glycerol is unknown, one must not lose sight of the possibility that these cells may be the source of glycerol (perhaps derived from fat) and other foodstuffs which the bacteria employ.

The significance of glucose. Because of the ubiquity of glucose in the body and its obvious importance in metabolism in most cells, its rôle in the nutrition of the tubercle bacillus cannot be neglected. Even the earlier workers like Proskauer and Beck (1894) were of the opinion that glucose improved growth in media containing glycerol. However there is a general belief that glucose cannot completely replace glycerol. Many strains will not grow when dependent for their carbon supply upon glucose alone.

For instance, S. Kondo (1925) found this to be the case with 3 out of 6 different strains. Frouin (1921) believes that high concentrations, e.g., 15 per cent, are necessary. In agreement with Long and Finner (1927) and with Merrill (1930) we have found that H37 will grow on 5 per cent glucose, but only scantily. Gamble and Herrick (1922) first showed for H37 and another human strain that glucose disappeared in the process. Merrill, in a much more thorough investigation employed glucose or levulose, at first in a broth (1930) and later in a synthetic medium where ammonium sulphate served as the source of nitrogen (1931a and b). He estimated the amount of carbohydrate disappearing, the carbon dioxide formed, and the ammonia produced.

From his data most of the glucose disappearance occurred because of oxidation rather than synthesis into protoplasm. The oxidation was complete, i.e., no intermediate product accumulated in the solution.

Our own results show that glucose can stimulate respiration, but in comparison with glycerol, very poorly. The stimulation can be observed at concentrations of 0.1 per cent which is the normal value in the blood, so that it may be significant for the life or growth of the tubercle bacillus in the body.

The position of sodium lactate. This is rather unique. We have found that for short periods it stimulates the respiration better than glycerol. We have not investigated its influence upon the bacteria over a period of more than 5 hours. For that reason it is impossible to emphasize the complete failure of 5 per cent sodium lactate to support the growth of H37. The inadequacy of sodium lactate for growth has been observed, among others, by Long (1922) and S. Kondo (1925). The early stimulation of growth in concentrations of 1 per cent lactate needs further study. At the present time attention may be directed to two facts. First, very low concentrations of lactate, even as low as the physiologic level in the blood, stimulated the respiration. This may furnish energy for survival of the bacteria. Second, the monocytes in which the organisms are usually situated can form large quantities of lactic acid from glucose. The former may be the superior source of energy, judging from the respiratory experiments. These facts may well have some bearing upon the parasitic relation between tubercle bacilli and epithelioid cells, evidence for which has been adduced by Cunningham, Sabin, Sugiyama, and Kindwall (1925).

The influence of glycogen. The stimulation of the respiration by glycogen shows that the tubercle bacillus has the power to utilize that substance. Corper and Sweany (1918), however, were unable to demonstrate diastase or sucrase in tubercle bacilli killed with toluol. The intermediate transformations are therefore quite unknown.

Soaps and fats. These have received a certain amount of attention in the literature, but mostly as inhibitors of growth.

Larsen, Cantwell and Hartzell (1919) added castor oil soap to glycerol broth in sufficient amount to depress the surface tension to 44 dynes (about 0.01 per cent) with the result that growth was delayed. Cooper (1930) added castor oil soap to Proskauer and Beck's medium and observed that growth was delayed in proportion to the concentration of soap and the depression of the surface tension. Soap in a concentration of 0.04 per cent or less, added to a medium similar to the above but containing no glycerol, gave no growth in three months. Chang (1922) observed with soaps of oleic, palmitic, and stearic acid and of castor oil, a depression but no suppression of growth. With the saturated soaps there was less effect than with the unsaturated. Schöbl (1924) in a most comprehensive study of this question found that concentrations of between 1 and 0.5 per cent of oleic, palmitic and stearic acid were inhibitory.

Our own results showing pronounced stimulation of respiration at very low concentrations make it very likely that they can furnish considerable energy. It is an open question whether this can be applied toward the creation of new protoplasm. Many of the earlier experiments upon growth seem to have been done upon fluid media where their results are invalidated by the surface-lowering activity of soaps which causes the cultures to sink. In our own experiments we found that concentrations of 0.1 per cent gave very marked stimulation of respiration. The failure of growth to occur on soap media of this strength made up in agar is not conclusive because there would not be growth even upon glycerol at such low concentration.

Lecithin. The availability of lecithin as a food is amply demonstrated by these experiments. Marpmann (1897) was able to show that a 10 per cent solution of lecithin which settled to gel supported growth. It is quite likely that the excellence of egg media for growth is due, in part, to the presence of large quantities of lecithin.

Cream and serum fat. The pronounced stimulation of respiration with the fat derived from cream and from serum furnished some indication that these are used as such or after hydrolysis. There is some work to support the second alternative. Wells and Corper (1912) showed that tubercle bacilli have an esterase which decomposes ethyl butyrate and glycerol triacetate. Michaelis and Nakahara (1923) found a lipase which was active in decomposing tributyrin.

Serum fat. Serum fat is one of at least three materials which occur in the blood in amounts that stimulate the respiration of H37, i.e., serum fat, glucose and lactate. To this list glycerol should probably be added. The powerful action of serum fat is quite outstanding. In vitro it accounts for most of the bacterial respiration in serum, and may affect the tubercle bacillus in equally striking fashion in the body.

Caseous material. The experiments with caseous material demonstrate that the tubercle bacillus, even in a relatively advanced lesion, has a possible source of energy in its immediate environment. Starvation of the bacteria does not therefore appear to be a factor in the arrest of tuberculosis. The substance or substances in the caseous material which supply the energy were not determined. The possibilities have been indicated by several authors.

Schmoll (1904) states that the bulk of caseous material consists of coagulated protein. He also found soap and glycerophosphoric acid. In caseous human nodes Caldwell (1919) found that 19.7 per cent of the ash-free dry residue was lipin. Of this 31 per cent was lecithin. Only traces of purin were found. In the caseous material obtained from bovine lymph nodes a lower value was obtained for lecithin (16 per cent) and a comparable one for cholesterol (27 per cent). The total nitrogen was 0.26 per cent of the dry weight of which proteose was 35 per cent, free amino acid 15 per cent, and amino acid 27 per cent.

From the results obtained by a study of the separate constituents of caseous material one would expect that it would be capable of supporting a high respiration unless a toxic substance was also present. For the existence of the latter there was no evidence during the periods embraced by these experiments.

SUMMARY

The tubercle bacilli of the H37 strain consume oxygen at a rate which is comparable to that of mammalian cells.

One of the foodstuffs which causes the highest oxygen consumption is glycerol. However, for purposes of oxidation this can be replaced by a number of substances. Some are about as effective as glycerol. These are sodium lactate, the sodium salts of the higher fatty acids, and diluted blood serum or caseous material. Lecithin, milk and serum fat fall in the same category. Smaller effects are observed with glucose and glycogen.

For respiration, it is not necessary that the substances mentioned should be present in the concentrations required for abundant growth. Stimulation of respiration by glycerol is perceptible at 0.005 per cent and by sodium lactate in nearly as weak solution. The effect of soap is very marked at 0.1 per cent. Milk fat at this dilution seems to produce detectable stimulation.

Both glucose and lactate are effective in concentrations at which they occur normally in the blood. These observations explain how it may be possible for the tubercle bacillus to obtain energy from the circulating blood in the body at concentrations of various foodstuffs which in culture media may be unable to support rich growths.

The rôle of the white blood cells in supplying the bacteria with foodstuff has only been suggested. Glycogen and lactate might be more readily furnished by monocytes, for instance, than by the circulating blood.

Finally, there has been a study of casous material. The latter contains several ingredients each of which is capable of supporting a high level of respiration by H37. In fact, upon this material there was evidence of adequate food supply and no toxic effect upon the respiration for several hours.

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